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(54) Title: IMPROVED PROKARYOTIC EXPRESSION OF PROTEIN

(57) Abstract

The invention herein described relates to a novel prokaryotic expression system and proteins expressed thereby. The regulated expression or alteration of the *wprA* gene from the genome of *B.subtilis* has been shown to result in enhanced secretion of selected native, heterologous or recombinant polypeptides. This alteration provides the means by which the yield of secreted polypeptide is increased compared to strains carrying a wild type copy of the *wprA* gene.

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IMPROVED PROKARYOTIC EXPRESSION OF PROTEIN

The invention relates to a novel prokaryotic expression system and proteins expressed thereby.

The industrial production of proteins has, in many instances, exploited the native expression and secretory systems of micro-organisms and specifically bacteria. For example, and without limitation, the bacterium *Bacillus subtilis* is known to produce and secrete a number of proteins. One of these proteins α-amylase is of industrial importance and therefore the harvesting of this secreted protein is an activity currently undertaken by industry. However, the yield of this protein is significantly reduced by protein degradation during or immediately after, or just after, passage through the cell membrane.

It therefore follows that there is a need to provide a protein expression system which enhances the production of native protein or indeed heterologous or recombinant protein and more specifically enhances this production by reducing the degradation of protein.

It is also known to provide microorganisms that express and, advantageously, secrete heterologous protein i.e. proteins that are not native to that particular bacteria. This sort of system typically involves transformation of a bacterial cell with heterologous DNA with a view to manufacturing or producing recombinant protein. Microorganisms such as *Escherichia coli* (bacteria) *Saccharomyces cerevisiae*, *Aspergillus nidulans* and *Neurospora crassa* (fungi) have been used in this fashion.

The expression of heterologous protein in these primitive eukaryotes also allows some desirable eukaryotic post translational modifications to occur in these heterologous proteins leading to increases in the stability of the expressed protein and subsequent improvements in yield. More recently the use of mammalian and insect cell culture systems have been developed to facilitate the expression of eukaryotic proteins that for various reasons cannot be expressed in a prokaryotic host cell.

However the cost effectiveness of producing recombinant protein still remains the major advantage offered by genetically engineered prokaryotic expression systems and indeed significant advances have been made in the development of genetically engineered *E.coli* strains that increase the yield of specific recombinant proteins. The evolution of these bacterial strains has also been married with an ever increasing development of more efficient vectors adapted to optimize the expression of recombinant protein. These vectors commonly contain promoter elements that can be switched on or off with ease.

However there are two major disadvantages when using *E.coli* as a means of expressing recombinant protein. Firstly the high levels of expression may lead to a precipitation of recombinant protein in the bacterial cytoplasm as "inclusion bodies". This feature was thought to be advantageous as it can provide a simple means of separating the insoluble recombinant protein from the soluble endogenous *E.coli* protein. However, in reality this advantage is not a general feature of the system as in many cases the protein remains as an insoluble precipitate that can only be released into solution by using strong chaotropic agents. This presents a major problem if the protein in question is particularly labile and therefore loses biochemical or biological activity upon denaturation. Secondly the expression of foreign protein in *E.coli* leads to the rapid

degradation of these proteins via an efficient proteolytic system. Therefore difficulties arise with regard to the isolation of intact recombinant protein from *E.coli* cells.

E. coli strains (TOPP series, BL21) have been engineered to allow the expression of recombinant proteins that would ordinarily be difficult to express in traditional laboratory strains of *E. coli*. However these engineered. *E.coli* strains are invariably not as biologically disabled as traditional laboratory strains of *E. coli* and as a consequence require containment levels that are higher than what would normally be required.

The identification of alternative prokaryotic host cells and the development of means that facilitate the production of soluble, intact and biologically active protein is obviously desirable. However, notably the number of potential prokaryotic host cells is huge.

With a view to producing a novel protein expression system we have chosen to genetically engineer, as our example, *Bacillus subtilis* in order to provide an expression system that overcomes the problems of yield associated with prior art systems. We have focused our attention on providing a bacterial expression system that produces and ideally secretes proteins (native and/or heterologous and/or recombinant) into the culture medium because this system enables purification of the manufactured protein due to the absence of contaminating endogenous bacterial proteins and other macromolecules.

A number of *B. subtilis* genes encoding secreted proteases have been identified. For example, without limitation, *aprE, nprE, bpf, mpr, epr, nprB* and *vpr* genes of B. subtilis encode extracellular proteases. These proteases are secreted into

the culture medium and deletion of them from the *B. subtilis* genome reduces extracellular protease activity to less than 1% of wild type strains. Despite this fact experimental evidence suggests that *B. subtilis* strains deficient for extracellular proteases still show significant loss of secretory protein production through proteolytic degradation.

The identification of a cell wall-associated protease has lead us to investigate whether it has a role in expression and secretion of native and/or heterologous and/or recombinant protein in *B. subtilis* and whether or not the protease plays a major role in determining the levels of secretory protein production.

The wall protease A (*wprA*) gene encodes a 96 kDa polypeptide containing a signal peptide, propeptide and a protease which upon synthesis and export gives rise to two cell wall-bound proteins, CWBP23 and CWBP52 (Figure 1). The CWBP52 polypeptide has protease activity that is inhibited with PMSF, an inhibitor of serine proteases. The deletion of *wprA* does not result in any noticeable phenotype in terms of growth rate, cell morphology, sporulation or motility.

The *wprA* is characterised by the following characteristics; the polypeptide is cell-wall associated and is expressed during both exponential and stationary growth phases.

We decided to investigate the phenotype of a *wprA* deletion strain with particular reference to the secretion of heterologous protein.

Using homologous recombination, we have generated a strain in which the *wprA* gene from the *B.subtilis* genome has been placed under the control of an inducible promoter. As mentioned above, this *wprA* controllable strain has no

apparent phenotype, even in the absence of an inducer. Surprisingly, however, when the yield of a native bacillary model protein, the α-amylase from *Bacillus licheniformis* (AmyL) was compared in wild-type and *wprA* null strains there was an approximate 25% increase in the amount of α-amylase detected at the end of exponential growth, see figure 2A. The yield increased further to 41% after prolonged incubation (Figure 3). Knocking out the *wprA* gene also resulted in an increase in the yield of an engineered α-amylase, AmyLQS50.5, when compared to the wild-type *wprA* strain (figure 2B). This suggests that switching off or deleting the *wprA* gene significantly enhances production of secreted homologous and heterologous proteins in *B. subtilis*.

The importance of the *wprA* gene with respect to the secretion of native, heterologous or recombinant protein is also shown by experiments that attempted to identify additional genes involved in the secretory pathway. We have used a *B. subtilis* strain, CJ278, transformed with a vector expressing a chimeric α amylase gene, please see materials and methods for details of the chimeric α amylase gene and *B.subtilis* strain construction. This strain showed decreased chimeric α-amylase secretion in comparison to the level of secretion by a wild type strain expressing wild type α amylase. We have used transposon mutagenesis using the mini-Tn 10 delivery vector pIC333. The integration of this transposon into the *B.subtilis* genome is random. The screen involved the identification of integration mutants that show elevated secretion of chimeric α-amylase. The mutant strains thus identified were analysed further by rescuing the plasmid DNA from the mutant strain and sequencing the flanking regions around the transposon integration site to determine the region of the *B.subtilis* genome in to which the transposon had integrated. The sequence of the plasmid rescued from the transposon mutant TK108 shows that the transposon had

inserted into the *wprA* gene at position 2059.

It is therefore apparent that the absence of the WprA facilitates the secretion of native , heterologous or recombinant protein from *B.subtilis*.

It is therefore an object of this invention to develop a means of expressing recombinant protein in a prokaryotic expression system that allows the production of polypeptides in a biologically active form and at a high concentration.

It is a further object of the invention to develop a prokaryotic expression system that enables the secretion of recombinant protein into the culture medium to facilitate the purification of intact recombinant protein that retains biological activity.

According to a first aspect of the invention there is provided a bacterial strain wherein the *wprA* gene or its corresponding promoter has been altered by deletion and/or insertion and/or mutation and/or substitution so that either production of the said gene product is prevented or the gene product is non-functional to the extent that the use of the strain to produce native, heterologous or recombinant protein is facilitated.

In a preferred embodiment of the invention said bacterial strain, prior to said alteration, is wild-type for said *wprA* gene.

In yet a further preferred embodiment said bacterial strain is a Gram-positive bacterial strain.

In yet a still further preferred embodiment said bacterial strain is of the genus

Bacillus.

Reference hereto the term bacterial strain includes reference to any bacterial strain but ideally a Gram-positive bacterial strain and, more ideally, but not obligatory, a bacterial strain of the genus *Bacillus*.

It will be apparent to those skilled in the art that where heterologous protein is to be produced the said bacterial strain will be transformed so as to include DNA encoding at least one selected native and/or heterologous and/or recombinant protein.

In a preferred embodiment of the invention the said strain is manipulated so that at least a part of the *wprA* gene is deleted. Ideally a significant amount of the gene is deleted, but in certain aspects of the invention the pre-sequence, or a part thereof, or alternatively, the pro-sequence, or a part thereof, or alternatively the serine protease sequence, or a part thereof is deleted. Alternatively, a selected alternative part of the gene may be deleted or a combination of selected parts may be deleted.

In an alternative embodiment of the invention genetic material may be inserted into the *wprA* gene at at least one selected location with a view to preventing expression of the gene or synthesis of at least a part of the functional protein product.

Alternatively, at least one selected point mutation may be provided in the said gene with a view to either preventing synthesis of the protein or preventing expression of the gene. For example, and without limitation, the reading frame of the gene may be altered so as to encode a stop codon thus preventing synthesis of a functional protein.

In a yet further preferred embodiment of the invention said *wprA* gene is altered by way of modification of an expression control sequence, ideally a promoter such that the promoter is made responsive to a specific signal, for example, the *wrpA* gene may be placed under the control of an inducible promoter such that expression of the *wrpA* gene product may be selectively controlled.

In a preferred embodiment of the invention the bacterial strain is of the genus *Bacillus* and is ideally the species *Bacillus subtilis* or its close relatives such as *B. amyloliquefaciens, B. licheniformis* and *B. stearothermophilus*.

It is noted that the *wprA* gene encodes a protein with specific domains attributed to its derived amino acid sequence. The polypeptide can be divided into a signal peptide, propeptide and protease polypeptide that have been accurately defined in Margot and Karamata (Microbiology 1996 142 3437-3444). The signal peptide is involved in targeting the *wprA* gene product to the secretory apparatus necessary for translocation across the cell membrane. The propeptide is likely to be a chaperone-type molecule involved in folding and maturation of the CWBP52 protein into a biologically active form. The propeptide is stable and may perform other important functions. The protease polypeptide will require the presence of both the pre and pro-sequences to be effectively targeted and to function efficiently.

According to a second aspect of the invention there is provided a bacterial strain, preferably of the genus *Bacillus*, and ideally of the species *B. subtilis* having a deletion in at least part of the sequence represented in Figure 1 from nucleic acid base pair +154 to +247 inclusive, or a corresponding part of a homologous gene.

In a preferred embodiment there is provided a *B. subtilis* strain deleted for a part of the *wprA* gene encoding the signal peptide of the coding sequence of the WprA precursor protein.

According to a third aspect of the invention there is provided a bacterial strain preferably of the genus *Bacillus* and ideally *B. subtilis*, having a deletion of, at least a part of, the sequence represented in Figure 1 from nucleic acid base pair +154 to +1392 inclusive, or a corresponding part of a homologous gene.

In a preferred embodiment the said bacterial strain may be modified, additionally or alternatively, by deletion of, at least part of, the DNA sequence presented in Figure 1 from +247 to +1392, or a corresponding part of a homologous gene.

In yet a further preferred embodiment said *B. subtilis* strain is deleted for that part of the *wprA* gene encoding, at least a part of, the polypeptide CWBP23.

According to a fourth aspect of the invention there is provided a bacterial strain preferably of the genus *Bacillus* and ideally the species *B. subtilis* having a deletion of, at least a part of, the sequence represented in figure 1 from nucleic acid base pair + 1392 to +2835 inclusive, or a corresponding part of a homologous gene.

In a preferred embodiment there is provided a *B. subtilis* strain deleted for that part for the *wprA* gene encoding, at least a part of, the CWBP52 polypeptide.

In yet a further preferred embodiment of the invention said bacterial strain is deleted for at least part of the sequence represented in Figure 1 from nucleic

acid base +247 to + 2835.

In yet a further preferred embodiment of the invention there is provided a *B.subtilis* strain deleted for part of the *wprA* gene encoding either or both the proposed propeptide (CWBP23) or the serine protease (CWBP52).

In yet a further preferred embodiment the said strain includes a deletion in that part of the gene encoding a polypeptide that is a serine protease.

In a further aspect of the invention there is provided a method for producing a desired polypeptide, wherein a microbial strain as indicated above is used for the production of said polypeptide by growing said strain under conditions conducive to the production of said polypeptide of interest, and recovering said polypeptide of interest.

It is envisaged that the polypeptide of interest may be endogenous or heterologous to the strain in question.

According to an embodiment of this aspect said microbial strain is used as a host into which a polynucleotide construct encoding said polypeptide of interest is brought in a functional manner by which said strain is capable of expressing said polypeptide.

The polynucleotide construct may be transferred into the strain by any method known in the art, such as transformation, conjugation, or protoplast transformation. The construct may be a plasmid or any other vector suitable for the specific method used for introducing said polynucleotide construct into the microbial cell.

In the cell said construct may be present on a plasmid or integrated into the chromosome of said strain. Furthermore, it may be present as a single copy or in multiple copies provided by either amplification or by multiple integrations.

The polypeptide may be a peptide or protein of any type, especially an industrial enzyme. Said enzyme may be any enzyme that can be produced in a strain according to the invention, such as a carbonyl hydrolase, carbohydrase, protease, lipase, amylase, cellulase, oxido reductase, glucoamylase, or esterase.

In essence the invention provides a bacterial strain, ideally a *B. subtilis* strain, altered by mutation, substitution, insertion or deletion either entirely or in part, for the *wprA* gene, or a homologue thereof, which gene encodes a cell wall-associated serine protease. It is surprising that given the presence of additional extracellular protease genes in *B. subtilis* that deletion of the single copy *wprA* gene should result in a significant effect on the production of both endogenous and heterologous recombinant protein.

An embodiment of the invention will now be described by way of example only with reference to the following figures wherein:

Figure 1 shows the nucleotide sequence of the region of the *B. subtilis* genome containing the *wprA* gene and amino acid sequence of its product WprA;

Figure 2A represents yields of α-amylase released into culture medium. Closed symbols represent growth and open symbols α-amylase activity. *B.subtilis* strains KS408 (■), KS408 *wprA* :: pMutin2 with (♦) or without (•) IPTG (10 mM);

.....shows precursor (p) and mature(m) immunoprecipitated from whole culture samples and the bottom panel mature AmyL (m) released into culture medium. Quantification by phospho-imaging of the different forms of AmyL at time intervals following the chase; AmyL precursor ( ) and mature AmyL in whole cultures samples (♦), and mature AmyL released into growth medium (•). The amount of each form of AmyL is expressed as a percentage of the total AmyL (precursor + mature) synthesized during the pulse;....

Figure 2B is similar to the experiment described in Figure 2A but the *B. subtilis* strains are expressing a recombinantly manufactured chimeric α-amylase (AmyLQS50.5). Experimental details relating to induction of the *wprA* gene product are as in Figure 2A and are described in detail in the materials and methods;

Figure 2C is a diagrammatic representation of the construction of a B.subtilis strain encoding an inducible *wprA* gene. Closed flags represent the native *wprA* promoter (P*wprA*) and open flags the IPTG- inducible promoter (Pspac). Ori Ec; *E.coli* origin of replication; A represents the sub-cloning of a *wprA* PCR 5' fragment into the BamH1 site in pMutin2; B represents a single cross-over event between pM2*wprA*FP and the *B.subtilis wprA* gene; C represents the integration of pM2*wprA*FP into the *B.subtilis* chromosome by homologous recombination; and D represents the structure of the *B.subtilis* chromosome after the integration event.

Figure 3 represents a comparison of AmyL production in a wild type *B.subtilis* strain and a strain with a *wprA* gene product under the control of an IPTG inducible promoter in the absence or presence of IPTG. Cultures of *B.subtilis* were grown to stationary phase and AmyL activity was compared during exponential growth phase and after approximately 30 hours in stationary phase;

Figure 4 represents the secretion kinetics of AmyL from exponetially growing *B.subtilis* in the presence and absence of the *wprA* gene products. Representative data from pulse-chase experiments carried out on strains KS408 and KS408*wprA* ::pMutin2+/- 10mM IPTG. (A) Autoradiographs of pulse-chased AmyL following immunoprecipitation and SDS-PAGE. The top panel

Figure 5 represents cell-associated degradation of AmyL as determined by subtracting the data for the released mature AmyL from that obtained in the whole culture samples. The amount of AmyL at each interval is expressed as a percentage of the maximum amount of AmyL (precursor + mature) synthesized during the pulse;

Figure 6 represents the stability of AmyL in spent culture medium at 4^0C. (A) α-Amylase activity at time intervals in the absence (■) or presence (♦) of 10mM EDTA. (B) western blots of AmyL in spent culture medium at time intervals in the absence and presence of 10mM EDTA; and

Figure 7 represents the transcriptional activity of the *wprA* gene using the *wprA*Δ-lacZ transcriptional fusion. Growth (closed symbols) and β-galactosidase activity(open symbols) were measured in cultures of *B.subtilis* KS408 (■) and KS408*wprA* ::pMutin2 with (♦) or without (•) IPTG (10mM)

Table 2 shows the production of the AmyL α-amylase by *B. subtilis* in the absence or presence of the *wprA* gene product in a very nutritious, industrial type medium in an extended batch fermentation. Each strain was grown for approximately 7 days at 37^0C and α amylase activity was measured in the supernatant at the end of this period. Experimental details are given in the materials and methods.

MATERIALS AND METHODS

The initial analysis of the involvement of the *wprA* gene product in secretion of endogenous and heterologous recombinant protein dealt with the construction of a *B. subtilis* strain in which the single copy *wprA* gene promoter was

substituted by an IPTG inducible promoter. In the absence of IPTG the expression of the *wprA* gene is repressed. Upon addition of IPTG the *wprA* gene is induced.

Alternatively the *wprA* gene can be entirely or partially deleted from the *B.subtilis* genome as detailed in preceding description and the following methods.

Bacterial strains

The bacterial strains used are shown in table 1.

Table 1. Bacterial strains

Strain	Comments
E. coli XL1-Blue	-
B.subtilis DN1885 xylR::pKS405B	Encodes chimeric α-amylase AmyLQS50.5
B. subtilis DN1885 xylR::pKS408	Encodes wild type α-amylase, AmyL
B. subtilis DN1885 xylR::pKS405B, wprA:: pM2 wprAFP.	B.subtilis DN1885 xylR:: pKS405B with IPTG-inducible wprA
B. subtilis DN1885 xylR:: pKS408	B. subtilis DN1885 xylR::pKS408

wprA::pM2 wprAFP	with IPTG-inducible wprA
B.subtilis DN1885 Novo Nordisk J.of Bacteriology 172:4315-4321, 1991	α-amylase (*amyE*) negative derivative of *B.subtilis* RUB200

Growth media

B.subtilis and *E.coli* were maintained on antibiotic medium number 3 (Difco) solidified with 1.5% w/v agar and containing 1% w/v soluble starch. Batch cultures were grown in 2xYT broth which contained; tryptone (1.6%w/v), yeast extract (1.0%w/v) and NaC1 (0.5%w/v). Where required antibiotics were included in the growth media at the following final concentrations: chloramphenicol g/ml, ampicillin g/ml and erythromycin g/ml. Xylb%w(/v) was added to induce the synthesis of α-amylase from a xylose-inducible promoter. The comparison of α-amylase production in B.subtilis wild type and a strain deleted for the *wprA* gene product was also done in an industrial type medium containing potato starch (100g/l), barley flour (50g/l), BAN 5000 SKB (0.1g/l), sodium caseinate (10g/l), soy bean meal (20g/l), Na_2HPO_4.12 H^20 (9g/l) and pluronic (0.1g/l). For the wild-type strain the medium was supplemented with 6 g/ml chloramphenicol and 0.2% xylose. For the *wprA*

deletion strain medium was supplemented with 6 g/ml chloramphenicol 5 g/ml erythromycin and 0.2% xylose.

DNA manipulations and bacterial transformation

Restriction digestion, DNA fragment purification, ligation and transformation of *E.coli* were carried out as described previously (Sambrook et al., 1989). Chromosomal DNA was isolated from *B.subtilis* using the IGi Genomic extraction kit (Immunogen International). PCR was carried out with Taq DNA polymerase (Appligene) using *B.subtilis* DN1885 chromosomal DNA as the template. Plasmid DNA was purified from *E.coli* and *B.subtilis* with the Tip-100 plasmid extraction kit (Qiagen). Oligonucleotide primers for PCR were synthesized using a Beckman Oligo 1000. *B.subtilis* was grown to competence and transformed with integrative plasmids.

α-Amylase assay

The quantity of secreted α-amylase was quantified using the Phadebas α-amylase assay kit (Kabi Pharmacia). The cells from culture samples were pelleted by microcentrifugation and the α-amylase activity in the supernatant determined as described by the manufacturer.

Construction of a Strain Encoding An Inducible *wprA*

To determine whether the products of the *wprA* gene are involved in the co-or post –translocational degradation of AmyL, we constructed a strain of *B.subtilis* in which an intact copy of the gene is under the control of the isopropylthio-β-D- galactoside (IPTG) –inducible Pspac promoter. The constructs were made using the pMutin2 integration vector. A 357-base pair DNA fragment

corresponding to the 5' end of the *wprA* gene was amplified by PCR from *B.subtilis* KS 408 chromosomal DNA using oligonucleotide primers WPR-F (5' GCGCGCGCGGATCCGGGATAACATGAAACGC 3') and WPR-R (5' GCGCGCGCGGATCCCCATCCTCCGCTGTG 3'). This fragment was cloned into the unique BamH1 restriction site of pMutin2 using *E.coli* XL1-Blue as the host.

The resultant plasmid, pM2*wprA*FP, was used to transform *B.subtilis* KS408 to produce strain KS408 *wprA* ::pMutin2. Since the *wprA* gene of KS408 *wprA* ::pMutin2 is under the control of the Pspac promoter, its expression can be controlled by the presence or absence of IPTG. Additionally, a transcriptional fusion (*wprA* Δ-lacZ) was created between the native *wprA* promoter and lacZ to allow the expression of *wprA* to be monitored via β-galactosidase activity, Figure 7.

Construction of a WprA Negative Strain By Deletion in the *B.subtilis* DN1885 *wprA* Gene

Plasmid pCJ791, encoding a N-terminal fragment (bp 133 to bp 615) and a C-terminal fragment (bp 2364 to bp 2781) of the wprA gene from *B.subtilis* DN1885, was constructed in four steps:

i) a 382 bp N-terminal fragment of the wprA gene was amplified by PCR from *B.subtilis* DN1885 using oligonucleotide primers
CLJe7
(5'-GGAATTCCAAAGCTGCAGCGGCCGGCGCG-3'),
and CLJe8
(5'-GAAGATCTCGTATACTTGGCTTCTGCAGCT-3').

This fragment had a EcoRI restriction site at the 5'-end and a BglII restriction site at the 3'-end. Simultaneously, a 419 bp C-terminal fragment of the *wprA* gene was amplified by PCR from *B. subtilis* DN1885 using oligonucleotide primers

CLJe9

(5'-AGATCTGGTCAACAAGCTGGAAAGCACTC-3')

and CLJe10

(5'-CCCAAGCTTCGTGACGTACAGCACCGTTCCGGC-3').

This C-terminal fragment had a BglII restriction site at the 5'-end and a HindIII restriction site at the 3'-end.

ii) The two DNA fragments, encoding the N- and C-terminal sequences of the *wprA* gene, were digested with BglII restriction enzyme and ligated to form a fragment of 801 bp. Using oligonucleotide primers CLJe7 and CLJe10, the 801 bp fragment was amplified by PCR from the ligation mixture. The amplified 801 bp fragment was digested with EcoRI and HindIII restriction enzymes.

iii) A 4.4 kbp EcoRI to HindIII fragment from plasmid pSJ2739 (described in patent application WO 96/23073, figure 6) was purified and used as vector for the 801 bp fragment. This plasmid is based on the pE194 origin of replication which means that the replication of the plasmid is temperature sensitive.

iv) The two EcoRI to HindIII fragments (801 bp and 4.4 kbp) were ligated and plasmid pCJ791 was obtained by selecting for resistance to erythromycin at 28°C using *B. subtilis* DN1885 as host strain.

Plasmid pCJ791 was integrated into the chromosome of *B.subtilis* DN1885 by selecting for resistance to erythromycin at 37°C. Since pCJ791 is based on the pE194 origin of replication, transformants were selected in which the plasmid had integrated into the chromosome by homologous single crossover recombination between one of the plasmid *wprA* sequence and the corresponding chromosomal *wprA* sequence. Two types of integrant strains could be the result of the integration event, i) the integrated plasmid followed by the wild-type *wprA* gene or ii) the wild-type *wprA* gene followed by the integrated plasmid. For the construction of a clean *wprA* deletion strain, both types of integrant strain could be used. Hence, the integration event was not investigated further.

A clean *wprA* deletion strain was then constructed by homologous single crossover resulting in release of the integrated plasmid. There were two ways in which the plasmid could be released from the chromosome i) by the same recombination as the plasmid was integrated or ii) by recombination between the sequence that was not involved in the integration event. For the first case, the resulting strain would have a wild-type *wprA* gene on the chromosome. If the second case occurs, the resulting strain would have a deleted *wprA* gene on the chromosome and, by that, the wanted event. In order to release the integrated plasmid, twelve transformants were inoculated in TY-medium without selection and cultivated at 28°C over night. The cultures were once again inoculated in fresh TY-medium and cultivated at 28°C over night. After three rounds of inoculation, the cultures were spread on LB-plates without selection and, subsequently, the obtained colonies were screened for sensitivity to erythromycin. Twenty-four colonies sensitive to erythromycin (Erm^s) were checked for the presence of a deleted *wprA* gene by PCR directly on colony

using oligonucleotide primers CLJe7 and CLJe10. Four of these Erm^s colonies had a deleted *wprA* gene on the chromosome. The authenticity of the *B. subtilis* DN1885Δ*wprA* strain was confirmed by Southern Blot Hybridisation.

Isolation of a *wprA* mutant with increased level of secreted chimeric α-amylase

In a previous study where the aim was to investigate how the net charge of proteins affected their passage through the negatively charged cell wall in *Bacillus subtilis* it was observed that both wild type AmyL (α-amylase from *Bacillus licheniformis*) and chimeric variants are subject to co-and/or post-translocational degradation. Protease(s) responsible for this degradation are likely to be associated with the cytoplasmic membrane or cell wall, since the proteolytic degradation occurs on the outer surface of the cytoplasmic membrane ("Construction and use of chimeric α-amylase to study protein secretion in *B.subtilis*" PhD thesis by Keith Stephenson, University of Newcastle Upon Tyne, 1996; " Secretion of chimeric α-amylase from *Bacillus subtilis*" PhD thesis by Christina Lund Jensen, Technical University of Denmark,1997). In a search of factors involved in this degradation, a screening system based on the *B.subtilis* strain CJ278, expressing the chimeric α-amylase (AmyLQS55-6) was set up. A mutant library was prepared by transposon mutagenesis, and subsequently screened for mutants with increased halo formation on amylase screening plates.

Construction of a chimeric α-amylase, AmyLQS55-6:

In the following an overview of the steps involved in the construction of the chimeric α-amylase AmyLQS55-6 is given. A detailed description is given in: "Secretion of chimeric α-amylases from *Bacillus subtilis*." Ph.D thesis by

Christina Lund Jensen, Technical University of Denmark, 1997.

The chimeric α-amylase, AmyLQS55-6 was constructed by swapping specific blocks of the mature portion of the α-amylase from *B. licheniformis* (AmyL) with the corresponding blocks from the α-amylase from *B.amyloliquefaciens* (AmyQ) or from *B.stearothermophilus* (AmyS). The individual DNA blocks were constructed by using a PCR-based in vitro gene splicing method, the SOE method (splicing by overlap extension, Horton et al. Gene 77, 61-68, 1989). The amyL gene has a unique Pst1 site located within the signal sequence and a unique HindIII site 3' to the transcription terminator. The amyLQS55-6 gene was therefore designed as in-frame PstI to HindIII DNA fragments encoding the mature part of the α-amylase. The amyLQS55-6 gene was divided into 3 separate DNA blocks, with block 1 covering a PstI-BamHI fragment, block 3 a KpnI-SalI fragment and block 4 a SalI-HindIII.

The properties of each block is given below, with the base numbers calculated in relation to the start codon in each gene.

Block 1: bp 79-132 amyL, bp 151-174 amyS, bp 157-198 amyQ, bp 199-213 amyL,

Block 3: bp 562-993 amyL, bp 1018-1095 amyS, bp 1072-1095 amyL

Block 4: bp 1096-1221 amyL, bp 1237-1419 amyS, bp 1411-1542 amyQ, bp 1537-1798 amyL

Between block 1 and 3 is a wild type amyL block covering bp 214-561.

The individual blocks were cloned into pUC19 in the correct order to produce the PstI to HindIII gene fragment encoding the mature AmyLQS55-6 protein.

For assembly of the individual blocks advantage was taken of the unique restrictions sites generated at their ends (created by the SOE method).

Expression of the chimeric α-amylase, amyLQS55-6

The amyLQS55-6 gene was integrated into the *B.subtilis* chromosome by homologous recombination between a plasmid encoded and a chromosomal encoded copy of the xylR-gene. The assembled amyLQS55-6 gene was cloned into plasmid pCJ92. Plasmid pCJ92 is derived from pSX63 which encode for a xylose-inducible promoter system (for detailed information about the construction of plasmid pCJ92; Secretion of chimeric α-amylases from *Bacillus subtilis*. Ph.D thesis by Christina Lund Jensen, Technical University of Denmark, 1997). The EcoRI to BglII fragment of the amyLQS55-6 gene encoded by pCJ92 was cloned into pUC19 EcoRI and BamH1 restriction sites in *E.coli* SJ2, resulting in plasmid pCJ272. Plasmid pCJ272 does not contain an origin of replication that is functional in *B.subtilis*. The integration plasmid, pCJ272, was introduced into the *B.subtilis* strain DN1885 and transformants were obtained by selection for chloramphenicol resistant colonies. The plasmid was integrated into the chromosome of DN1885 by a single, homologous recombination between the plasmid encoded and chromosomally encoded copies of the xylR-gene.

The integration of the α-amylase expression cassette into the chromosome of DN1885 resulted in a stable system which allowed the production of α-amylase to be induced in the presence of xylose.

Screening system:

The screening system for identification of mutants with an improved secretion

of α-amylase is based on the *B.subtilis* strain CJ278 (DN1885 xylR::pCJ272) harbouring the gene encoding the chimeric α-amylase AmyLQS55-6. In comparison to the wild type AmyL, the level of α-amylase secretion from strain CJ278 is about 1%, meaning that CJ278 gives rise to colonies with a small and well-defined halo of starch degradation on plates. Therefore, it was considered an ideal candidate for screening of yield mutants.

Mutagenesis protocol:

For transposon mutagenesis of strain CJ278, the mini-Tn10 delivery vector pIC333 was used (Steinmetz, M. and Richter, R. 1994. J. Bacteriol. 172:5019). Outside the transposon, this plasmid carries a modified transposase gene conferring relaxed target specificity, a thermosensitive origin of replication and an erythromycin resistance gene for selection at permissive temperatures. The 2.2 kbp transposon encodes the spectinomycin resistance gene and the pUC8 origin of replication, allowing replication in *E.coli*. Plasmid pIC333 was transformed into strain CJ278 and erythromycin resistant transformants were inoculated to TY-medium supplemented with 0.4% glucose and spectinomycin (120 μg/ml) and grown over night at 28°C. The over night culture was diluted 1/100 in TY-medium supplemented with 0.4% glucose and spectinomycin (120 μg/ml). After 3 hours of cultivation the temperature was shifted to 37°C (which is the restrictive temperature) and the culture was cultivated for an additional 4 hours. Aliquots of the culture were plated on LB-amylopectin (coupled to Cibacrone red) plates supplemented with 0.4% glucose, 0.01 M phosphate pH 7, 0.2% xylose and 120 μg/ml spectinomycin and incubated over night at 37°C. Colonies with a distinctly larger halo, indicating a higher amount of secreted α-amylase, appeared with a frequency of 1/150. One such transposon mutant,

forming a larger halo of starch degradation than the parent strain, was strain TK108.

The mini-Tn10 transposon and its flanking regions from strain TK108 were rescued, taking advantage of the pUC origin of replication present in the transposon. The TK108 chromosome was totally digested with EcoRI, and religated with T4 DNA ligase. The ligation mixture was transformed into *E.coli* SJ2 (Diderichsen et al, 1990. J. Bacteriology 172, 4315-4321), selecting for spectinomycin resistance. Plasmid DNA from spectinomycin-resistant transformants was used for DNA sequencing. The DNA sequences was determined by the dideoxy chain termination method (Sanger et al 1997) and by using mini-Tn10 specific primers: 5'- CCA ATA CGC AAA CGC CCT CTC-3' and 5'- TAG TGA CAT TTG CAT GCT TC- 3', which correspond to position 137-117 and 2181-2200, respectively, on the mini-Tn10 transposon sequence.

The sequence of the plasmid rescued from transposon mutant TK108 shows that the transposon had inserted into the *wprA* gene, at position 2059.

Results and Discussion

The development of efficient alternative methods of native, heterologous or recombinant protein manufacture is obviously desirable. It is apparent that not all heterologous protein can be produced in a soluble, biologically active form therefore methods that facilitate the production of such proteins are continuously being designed.

We have taken the approach to develop the genus *Bacillus* and its close relatives as an alternative host cell for the production of the both native and heterologous

and recombinant protein. These bacteria have considerable advantages over other species due to their ease of growth in batch cultures and their rapid rate of cell division and furthermore their ability to secrete proteins into the culture medium at high concentrations. Furthermore, we have decided to focus on the development of an expression system that produces secreted soluble protein into the culture medium to facilitate the purification of such proteins from contaminating endogenous bacterial proteins and other macromolecules. A major problem with this methodology is that many bacterial systems actively secrete proteases into the culture medium that degrade proteins in the immediate environment of the bacterial cells. Some *B.subtilis* strains have been engineered to delete these genes from the bacterial genome to reduce the loss of protein through proteolytic activity. However, this can still lead to reduced yields of intact protein due to, amongst other things , the release of intracellular proteases into the growth media as strains that are multiply deficient in extracellular proteases become prone to lysis thereby releasing cellular contents into the surrounding growth media.

Additionally, we have undertaken pulse-chase labelling experiments to identify the sites at which proteolysis of secreted proteins occurs. The amount of mature Amy L (a model secreted protein) released into the culture medium can be seen to increase with time until it reaches a constant level. This level represents only approximately 25% of the total Amy L synthesized, an amount consistent with the AmyL remaining in whole culture samples. This means that 75% of the initially synthesized AmyL is degraded. By determining the proportion of AmyL that remains cell-associated at each time point after the addition of the chase solution, by subtracting the amount of AmyL released from that observed in whole culture samples, it was possible to determine that AmyL degradation

occurred in a cell-associated location and within 7 minutes of the addition of chase, Figure 6. This data suggests that the observed degradation of AmyL occurs during or shortly after translocation across the membrane and in a cell-associated location.

The *wprA* gene of *B. subtilis* encodes a cell wall-associated serine protease. The *wprA* gene product is composed of a presequence (signal peptide) to assist in targeting the protease to the secretory apparatus, a prosequence which produces a stable 23kDa protein product most likely with chaperone type activity and a 52kDa serine protease. We have engineered the *wprA* gene by placing it under the control of an IPTG inducible promoter element. This allows the expression of *wprA* to be strictly regulated, simply by the presence or absence of IPTG in the growth medium. When a *B.subtilis* strain which showed decreased α-amylase production is randomly mutagenised with the mini Tn 10 transposon, integrant mutants with enhanced α-amylase production were identified. Sequence analysis of rescued Tn 10 DNA revealed the integration site to be the *wprA* gene. We have also created a *B.subtilis* strain that has been genetically engineered to completely delete the *wprA* gene from the genome.

We have taken wild type *B. subtilis* strain DN1885 xylR::pKS405B transformed with a chimeric α-amylase gene and strain DN1885 xylR::pKS408 which is transformed with wild-type α-amylase and replaced the *wprA* promoter sequence with an IPTG inducible promoter contained in the plasmid pM2*wprA*FP (see materials and methods).

The activity of secreted wild-type α-amylase was assessed in *B. subtilis* cultures in the presence or absence of 10mM IPTG. A culture in which *wprA* was expressed from its native promoter was used as a controlled culture. Figure 2A

indicates that the growth rate and kinetics of a *B. subtilis* strain is not significantly affected by the absence of WprA protein (no IPTG added), as measured by culture optical density. However, in the absence of 10mM IPTG there is approximately a 25% increase in α-amylase activity in the culture medium compared to the wild-type strain in which the *wprA* gene is expressed from its native promoter (Figure 2A). Figure 2B indicates a comparable effect on the production of a chimeric -amylase.

The yield of native α-amylase in the culture medium was also assessed in stationary phase cultures of *B. subtilis* wild-type or IPTG-inducible *wprA* genes, (figure 3). The strains were grown for approximately 39 hours at which time cultures had been in stationary phase for approximately 30 hours. In the absence of IPTG the yield of α-amylase in the culture medium had increased by approximately 40% when compared to a strain expressing *wprA* from its native promoter. In contrast, the yield of α-amylase from KS408 *wprA* ::pMutin2 in the presence of IPTG (*wprA* on) was lower and on transition to stationary phase the yield of α-amylase was 95% that of KS408.

Additionally, when strains are grown in a rich industrial type medium in an extended batch fermentation culture, there is approximately a 78% increase in α-amylase activity in the absence of the WprA protein.

These data demonstrate that expression of *wprA* markedly influences the yield of released α-amylase.

We have additionally used coupled pulse-chase and immunoprecipitation techniques to investigate the secretion kinetics of AmyL in KS408 and KS408 *wprA*::pMutin2. Cultures were grown to exponential phase ($OD_{600} \sim 0.6$) and

pulse-chased with L-(^{35}S) methionine. Following inmmunoprecipitation and subsequent SDS-PAGE, both precursor and mature forms of AmyL were visualised by autoradiography, Figure 4. In the case of KS408, the processing of the AmyL precursor to the mature form was rapid; in samples taken immediately following the chase (0 min) only 27% of the total AmyL (precursor + mature) synthesized during the pulse was in precursor form, Figure 4. Processing was complete by 5 min post-chase when all the α-amylase was in mature form. The amount of mature AmyL in the whole culture sample (cells + growth medium peaked at 1 minute, after which time it declined until it reached a constant level of approximately 25% of the maximum detected, representing a significant loss of newly synthesized α-amylase during or shortly after translocation across the cytoplasmic membrane.

The involvement of the WprA protein in the secretion of protein from *B.subtilis* is also confirmed by the mutant screen undertaken to identify mutated strains that show enhanced secretion of a chimeric α-amylase. The mini-Tn 10 transposon randomly integrates into bacterial genomic DNA thereby creating insertional mutations if the transposon does not integrate in an essential gene. The mutant strain TK108 shows increased secretion of chimeric α-amylase when compared to a wild type control strain. The mini-Tn 10 delivery vector was recovered from TK108 genomic DNA and the flanking regions surrounding the vector was sequenced to determine the site of integration. The transposon had integrated at position 2059 of the *wprA* gene. The disrupted *B.subtilis* strain TK108, showed elevated α amylase secretion as monitored by the size of the halo produced around TK108 when compared to CJ278(wild type) on starch agar plates.

In conclusion we have shown that switching off or disabling or deleting the

single copy *wprA* gene significantly increases the yield in the culture medium of native, heterologous or recombinant proteins from *B. subtilis*. Importantly *B. subtilis* extracellular proteases are still actively secreted into the growth medium indicating that a major contributing factor in the production of increased yields of secreted α-amylase is removal of the cell wall associated protease encoded by the *wprA* gene.

Table 2

Relative yields of α-amylase is wild-type and *wprA* IPTG-inducible *B. subtilis* strains.

B. subtilis DN1885 xylR::pKS408	*B. subtilis* DN1885 xylR::pKS408 *wprA*::pM2 wprAFP
100	178

CLAIMS

1. A method for producing a native and/or heterologous and/or recombinant polypeptide from a microbial strain having a deletion and/or insertion and/or mutation and/or substitution in a *wprA* gene or its corresponding promoter such that the production and/or function of the gene product encoded by the *wprA* gene is affected in a manner that facilitates production of said polypeptide comprising;

i growing said strain under conditions conducive to the production and secretion of said polypeptide(s); and

ii. recovering said polypeptide(s) from growth media and/or said bacterial strain.

2. A method according to Claim 1 wherein said microbial strain is a Gram positive strain.

3. A method according to Claims 1 or 2 wherein said microbial strain is of the genus *Bacillus*.

4. A method according to Claim 1-3 wherein said bacterial strain is selected from *B.subtilis*, *B.amyloliquifaciens*, *B.licheniformis*, *B.stearothermophilus*.

5 A method according to Claim 1-4 wherein said polypeptide is selected from carbonyl hydrolase, carbohydrase, protease, lipase, amylase, cellulase, oxidoreductase, glucoamylase or esterase.

6. A bacterial strain wherein a *wprA* gene has been altered by deletion and/or insertion and/or mutation and/or substitution so that the production and/or function of the gene product encoded by said *wprA* gene is prevented to the extent that the production of native, heterologous or recombinant protein is facilitated.

7. A bacterial strain according to Claim 6 wherein said bacterial strain, before said alteration, is wild type for said *wprA* gene.

8. A bacterial strain according to Claims 6 or 7 wherein said bacterial strain is a Gram positive strain.

9 A bacterial strain according to Claims 6-8 wherein said bacterial strain is of the genus *Bacillus*.

10 A bacterial strain according to any proceeding Claim wherein said alteration is an insertion of genetic material into said *wprA* gene to prevent expression of said gene or to prevent synthesis of at least part of the functional protein product.

11. A bacterial strain according to Claims 6-9 wherein said *wprA* gene is mutated by at least one selected point mutation to prevent expression of either the *wprA* gene or the WprA protein.

12 A bacterial strain according to Claims 6-9 wherein said alteration is to an expression control sequence.

13. A bacterial strain according to Claim 12 wherein said expression control sequence is a promoter.

14. A bacterial strain according to Claim 13 wherein said alteration results in the provision of an inducible promoter to selectively control expression of the *wprA* gene.

15. A bacterial strain according to Claims 6-9, wherein said bacterial strain is *B.subtilis*, containing a deletion of at least part of the sequence represented in Figure 1 from nucleic acid base +154 to +247.

16. A bacterial strain according to Claim 15 wherein said *Bacillus* strain is deleted for part of the *wprA* gene encoding the signal sequence of the WprA precursor protein.

17. A bacterial strain according to Claims 6-9, wherein said bacterial strain is *B.subtilis*, containing a deletion of at least part of the sequence represented in Figure 1 from nucleic acid base +154 to +1392.

18. A bacterial strain according to Claims 6-9 or 17 wherein said deletion comprises at least part of the sequence represented in Figure 1 from nucleic acid base +247 to +1392.

19. A bacterial strain according to Claims 18 wherein the *wprA* gene is deleted for at least part of the *wprA* gene encoding CWPB23.

20. A bacterial strain according to Claims 6-9 wherein said bacterial strain is *B.subtilis*, containing a deletion of at least part of the sequence represented in Figure 1 from nucleic acid base +1392 to +2835.

21. A bacterial strain according to Claim 20 wherein the *wprA* gene is deleted for at least part of the *wprA* gene encoding CWPB52.

22. A bacterial strain according to Claims 20 or 21 wherein the *wprA* gene is deleted for at least part of the *wprA* gene encoding a serine protease.

23. A bacterial strain according to Claims 6-9 wherein said bacterial strain is *B.subtilis*, containing a deletion of at least part of the sequence represented in Figure 1 from nucleic acid base +247 to + 2835.

24. A bacterial strain according to Claim 23 wherein said bacterial strain is deleted for part of the *wprA* gene encoding either or both the propeptide or protease.

Figure 1


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AAC CCG GCT CTT TGA TAG AGC TGG TTT TTT TTA TAT TTA TCC CCT CAT ATT CCA AAT CAT   60
TTA AAA TAA CCT TAA AAT TCC CTG TAA GCG GTA TCT CGT CCT ATG AAA TTA TGA TAC CTT  120
CAA GGA GAT TCA TTA TTT TGC AGG AGG GAT AAC ATG AAA CGC AGA AAA TTC AGC TCG GTT  180

M  K  R  R  K  F  S  S  V

GTG GCG GCA GTG CTT ATT TTT GCA CTG ATT TTC AGC CTT TTT TCT CCG GGA ACC AAA GCT  240

 V  A  A  A  V  L  L  I  F  A  L  I  F  S  L  F  S  P  G  T  K  A
GCA GCG GCC GGC GCG ATC GAT CAG GCG GCT GCT CTG GAA AAC GGC AAA GAG CAG ACA GGC  300
 A  A  A  G  A  I  D  Q  A  A  A  L  E  N  G  K  E  Q  T  G
GCC ATG AAG GAG CCG GAA CAG GTG AAA TGG TAC AAA GTG ACC CCG GGA GCA ACG GAT ATT  360
 A  M  K  E  P  E  Q  V  K  W  Y  K  V  T  P  G  A  T  D  I
CAG AAA AAC TCA CAT ATG GCA CTG ACC GTA AAG AGT GAT TCA GTA CTG AAT GTA TCT GTA  420
 Q  K  N  S  H  M  A  L  T  V  K  S  D  S  V  L  N  V  S  V
TAT CCA AGT AAG GAA AAA GCG CTT AAA GAT GAG ACG TTT GAG ATG TAC CGT TCT TTC ACA  480
 Y  P  S  K  E  K  A  L  K  D  E  T  F  E  M  Y  R  S  F  T
GCG GAG GAT GGA AAA AGC GAA GTC ATT TTT CCA TAC GCG TGG AGC GGC CCT TAC TAT GTA  540
 A  E  D  G  K  S  E  V  I  F  P  Y  A  W  S  G  P  Y  Y  V
AAA GTT GAA TAC CTC GGA GAA GAA GAA CCA GAG GAC GGC GGA ACG GCA GAA GCA GCT GCA  600
 K  V  E  Y  L  G  E  E  E  P  E  D  G  G  T  A  E  A  A  A
GAA GCC AAG TAT ACG ATT GGG TAT AAA GGC ACC AAA AAA CAG CCG TCA GAT TTA GAA GAG  660
 E  A  K  Y  T  I  G  Y  K  G  T  K  K  Q  P  S  D  L  E  E
GAA GAA GCT TGT CCG GTT GAA ATG AGT GTC GAT CAG AAG AAA TCA GGA AAA GGC ATC CTG  720
 E  E  A  C  P  V  E  M  S  V  D  Q  K  K  S  G  K  G  I  L
GAT AAG TTG AGA TCG ATT CGT GAT GAG CAG CTG AGC CAA ACA GCA GAA GGC AAA GAA CTG  780
 D  K  L  R  S  I  R  D  E  Q  L  S  Q  T  A  E  G  K  E  L
ACA AGC CTT TAT TAC AAA GCA GCA CCG TTT ATC GTT GCA AAG CTC GCA CTC AAT AAA ACA  840
 T  S  L  Y  Y  K  A  A  P  F  I  V  A  K  L  A  L  N  K  T
GCA AGA AAT GAA ATC TAT CAG GAT CTT GTG ACA TTA AAG CCG TTA TTT GAC GAT GTG TCA  900
 A  R  N  E  I  Y  Q  D  L  V  T  L  K  P  L  F  D  D  V  S
GAA AAC GGA GCA TCA TCT TCG TAT AAG GTC ACT GAA AAG GAT CAA AAA GCG ATC AAC CGG  960
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Figure 1-2 continued

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  E   N   G   A   S   S   S   Y   K   V   T   E   K   D   Q   K   A   I   N   R
CTA TAT GAT AAA GCT TTA CAA TCA GTC CCG TCA TTC CTT AAA GAG GAG ATA AAG AAA CAA 1020
  L   Y   D   K   A   L   Q   S   V   P   S   F   L   K   E   E   I   K   K   Q
GCG GAC CGA CTA AAT ATG AAG CAG CTG CAA GGC AAA ACA GCA GGA GCC ATT TTA ACA GAA 1080
  A   D   R   L   N   M   K   Q   L   Q   G   K   T   A   G   A   I   L   T   E
AAC AAT ATT GCA GCA AAA AGT GAA GTT CAG ACA ACA AAG GTT ATT TTC AAG GTG AAG GAC 1140
  N   N   I   A   A   K   S   E   V   Q   T   T   K   V   I   F   K   V   K   D
AAT AAA AGC CTC TCA TCC GTA CAT AAT GAA ATG AAG GGC TTT TCT GCA AGC GCG CAA TCG 1200
  N   K   S   L   S   S   V   H   N   E   M   K   G   F   S   A   S   A   Q   S
AAA AAA GAC ATA TCT AAT GTG AAA AAG GCA AAG AAA CTG TTT GAC AAT CTG TAT TCA TTT 1260
  K   K   D   I   S   N   V   K   K   A   K   K   L   F   D   N   L   Y   S   F
GAA CTT CCG AAA GAC GAG AAA CAG AAC GGC GCA TAT ACG GCA AGC GCC AAA AGG GTC AAA 1320
  E   L   P   K   D   E   K   Q   N   G   A   Y   T   A   S   A   K   R   V   K
AGC GCT GCT GCG ACA CTA TCC AAG ATG TCC AAT GTA GAG TTT GCG GAA CCC GTA CAG GAA 1380
  S   A   A   A   T   L   S   K   M   S   N   V   E   F   A   E   P   V   Q   E
TAC AAA AGC TTG GCA AAC GAT ATT CAG TAC CCT TAT CAA TGG CCG CTT AAA AAC AAC GGT 1440
  Y   K   S   L   A   N   D   I   Q   Y   P   Y   Q   W   P   L   K   N   N   G
GAA AAC GGC GGT GTC AAA AAT GCG GAT GTG AAA TAT GAG CCT GCC AAC ACA TTG CTG TCC 1500
  E   N   G   G   V   K   N   A   D   V   K   Y   E   P   A   N   T   L   L   S
AAA CGC AAG CTT AAC GAT ACA CTC ATT GCA GTA GTA GAC ACA GGC GTA GAC AGC ACG CTT 1560
  K   R   K   L   N   D   T   L   I   A   V   V   D   T   G   V   D   S   T   L
GCC GAT TTA AAA GGA AAA GTA AGA ACA GAT CTC GGA CAC AAT TTT GTC GGA CGA AAT AAC 1620
  A   D   L   K   G   K   V   R   T   D   L   G   H   N   F   V   G   R   N   N
AAT GCA ATG GAT GAT CAG GGG CAT GGG ACG CAT GTC GCA GGC ATT ATT GCA GCC CAA AGC 1680
  N   A   M   D   D   Q   G   H   G   T   H   V   A   G   I   I   A   A   Q   S
GAT AAC GGC TAT TCA ATG ACT GGA TTG AAT GCC AAA GCA AAA ATC ATC CCT GTA AAA GTG 1740
  D   N   G   Y   S   M   T   G   L   N   A   K   A   K   I   I   P   V   K   V
CTT GAT TCC GCA GGT TCC GGA GAT ACT GAA CAA ATT GCT CTC GGC ATC AAA TAT GCT GCT 1800
  L   D   S   A   G   S   G   D   T   E   Q   I   A   L   G   I   K   Y   A   A
GAC AAA GGA GCA AAG GTG ATT AAT TTA AGT TTA GGC GGA GGC TAC AGC CGC GTG CTT GAA 1860
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Figure 1-3 continued

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  D  K  G  A  K  V  I  N  L  S  L  G  G  G  Y  S  R  V  L  E
TTT GCT TTG AAG TAC GCA GCT GAC AAA AAT GTC TTG ATT GCC GCA GCC AGC GGG AAT GAT 1920
  F  A  L  K  Y  A  A  D  K  N  V  L  I  A  A  A  S  G  N  D
GGA GAA AAT GCC TTA TCT TAT CCT GCA TCT TCT AAA TAT GTG ATG TCA GTC GGC GCA ACG 1980
  G  E  N  A  L  S  Y  P  A  S  S  K  Y  V  M  S  V  G  A  T
AAT CGC ATG GAT ATG ACC GCT GAT TTC TCT AAC TAT GGA AAA GGT CTG GAC ATC TCT GCT 2040
  N  R  M  D  M  T  A  D  F  S  N  Y  G  K  G  L  D  I  S  A
CCA GGG TCT GAT ATC CCG AGC TTA GTG CCG AAC GGA AAT GTC ACG TAC ATG AGC GGA ACG 2100
  P  G  S  D  I  P  S  L  V  P  N  G  N  V  T  Y  M  S  G  T
TCT ATG GCG ACG CCA TAT GCT GCC GCC GCT GCG GGA CTG CTG TTT GCT CAA AAT CCT AAG 2160
  S  M  A  T  P  Y  A  A  A  A  A  G  L  L  F  A  Q  N  P  K
CTA AAA AGA ACA GAA GTT GAG GAT ATG TTG AAA AAG ACG GCA GAT ATT TCC TTT GAA GAA 2220
  L  K  R  T  E  V  E  D  M  L  K  K  T  A  D  D  I  S  F  E
AGT GTC GAT GGC GGA GAA GAG TTG TAT GAC GAT TAT GGC GAT CCG ATT GAA ATT CCG GAA 2280
  S  V  D  G  G  E  E  E  L  Y  D  D  Y  G  D  P  I  E  I  P
AAG ACA CCT GGT GTA GAC TGG CAT TCA GGC TAC GGG CGG CTG AAT GTC ATG AAG GCT GTC 2340
  K  T  P  G  V  D  W  H  S  G  Y  G  R  L  N  V  M  K  A  V
AGC GCA GCT GAT TTA CAG CTT AAG GTC AAC AAG CTG GAA AGC ACT CAA ACA GCT GTC AGA 2400
  S  A  A  D  L  Q  L  K  V  N  K  L  E  S  T  Q  T  A  V  R
GGA AGC GCG AAG GAA GGC ACC CTT ATC GAG GTG ATG AAC GGC AAA AAG AAA CTC GGC AGC 2460
  G  S  A  K  E  G  T  L  I  E  V  M  N  G  K  K  K  L  G  S
GCC AAA GCC GGA AAA GAC AAT GCG TTC AAG GTG AAT ATC GCG ACT CAA AAA CAG GAT CAA 2520
  A  K  A  G  K  D  N  A  F  K  V  N  I  A  T  Q  K  Q  D  Q
GTA CTG TAT CTG AAA GCA ACA AAA GGC GAT GCG AAA ACA TCG TAT AAA GTT GTC GTC GTC 2580
  V  L  Y  L  K  A  T  K  G  D  A  K  T  S  Y  K  V  V  V  V
AAA GGA AAA CCT TCC GGC ACA CCG AAA GTA AAC GCG GTG AAA ACG GAT ACG GCA GTA 2640
  K  G  K  P  S  G  T  P  K  V  N  A  V  K  T  K  D  T  A  V
AAA GGG AAG GCA AAC AGC AAA GCG ATG ATC AGA GTG AAA AAC AAA TCA AAG AAA GTC ATT 2700
  K  G  K  A  N  S  K  A  M  I  R  V  K  N  K  S  K  K  V  I
GCT TCT GCC AAA GCT GAC GCA AAA GGA ACG TTT TCG GTG AAA ATC AAA AAA CAA AAA GCC 2760
```

Figure 1-4 contiuned


```
 A  S  A  K  A  D  A  K  G  T  F  S  V  K  I  K  K  Q  K  A
GGA ACG GTG CTG TAC GTC ACG GCT GTG GAT ACA GAT AAA AAA GAA AGC AAG GAA GCA AAA 2820
 G  T  V  L  Y  V  T  A  V  D  T  D  K  K  E  S  K  E  A  K
GTT GTT GTT GAA AAG TAA CCA AAA AGC GGT GCT CGA TGC ACC GCT TTT TTA TTT GCG CCC 2880
 V  V  V  E  K
CCG TTG GAC TGC TGT GTA CAT AAT GGA TCG CTT TCC GTT TGA AGC CGT CCA TTC AAA ACG 2940
GTC TCC TGT CCC TTC GCT GCT GTA GTT GTA GTG ATG CTG CAT CGC GCA TTG TGC GGT GTC 3000
ATT TGA AAA GTG ATA AAA GGA CAC ACC CTG CCC CGC CGA AGT ATT CGA AAA GAC TCC TTT 3060
ATT ATA AGA AAA GAC GCA ATC GAA TAC CGA TAA GCC GAA GCT GTG CAA GTG CTG CAG 3117
```

Figure 2A

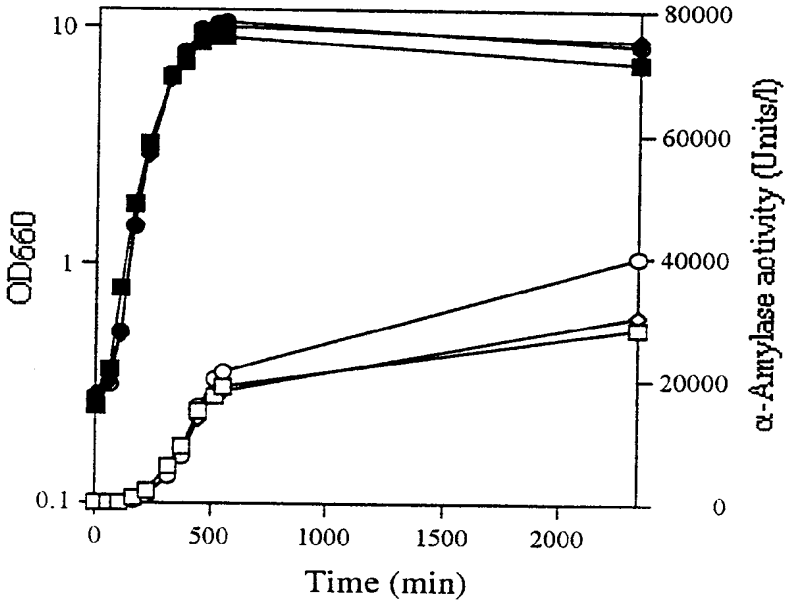

10
1
0.1
OD660
80000
60000
40000
20000
0
α-Amylase activity (Units/l)
0
500
1000
1500
2000
Time (min)

Figure 2B

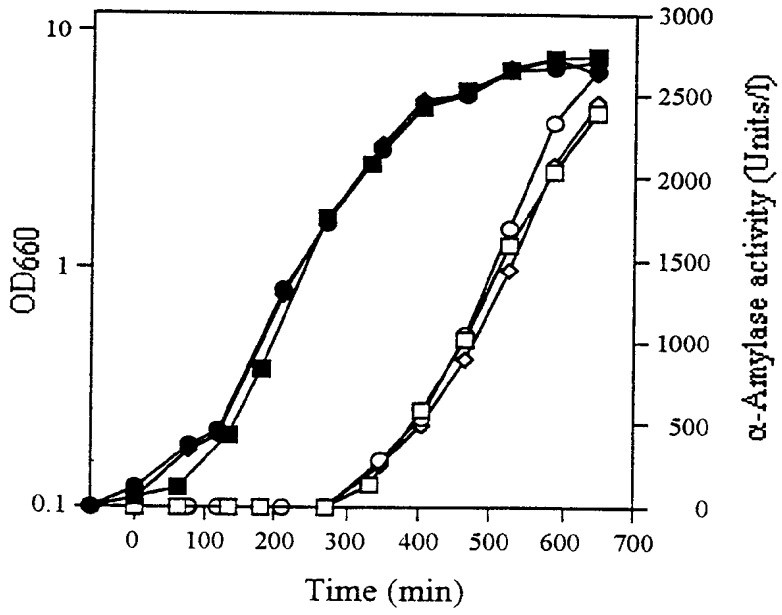

10
1
0.1
OD660
3000
2500
2000
1500
1000
500
0
α-Amylase activity (Units/l)
0
100
200
300
400
500
600
700
Time (min)

Figure 2C

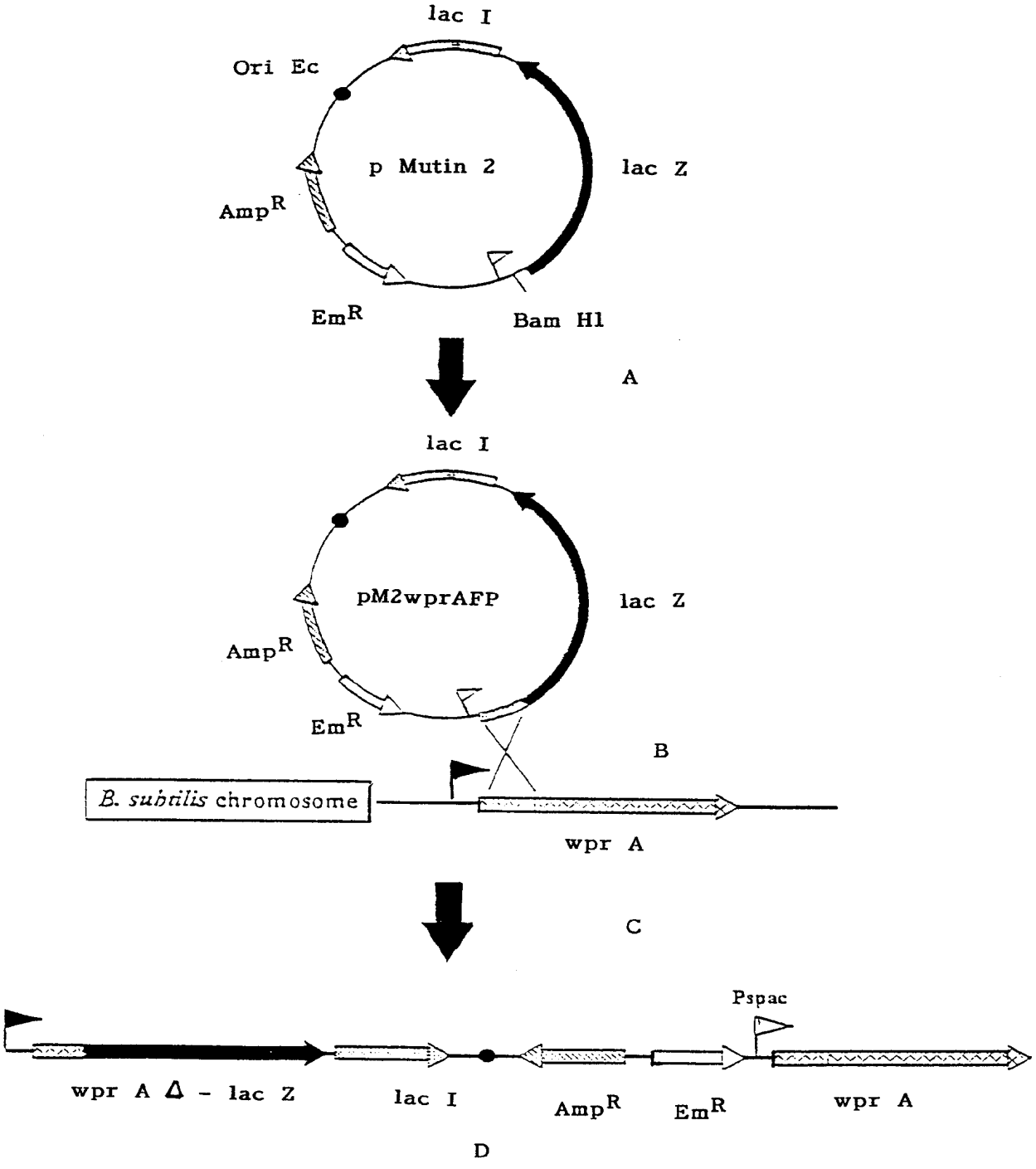
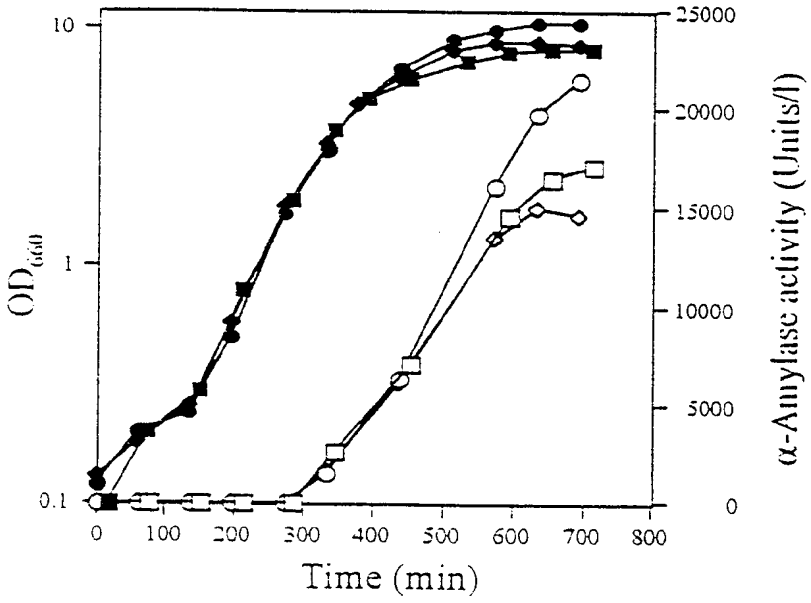

lac I
Ori Ec
p Mutin 2
lac Z
Amp^R
Em^R
Bam Hl
A
lac I
pM2wprAFP
lac Z
Amp^R
Em^R
B
B. subtilis chromosome
wpr A
C
Pspac
wpr A Δ - lac Z
lac I
Amp^R
Em^R
wpr A
D

Figure 3


10
1
0.1
OD_{660}
25000
20000
15000
10000
5000
0
α-Amylase activity (Units/l)
0
100
200
300
400
500
600
700
800
Time (min)

9/12 Figure 4

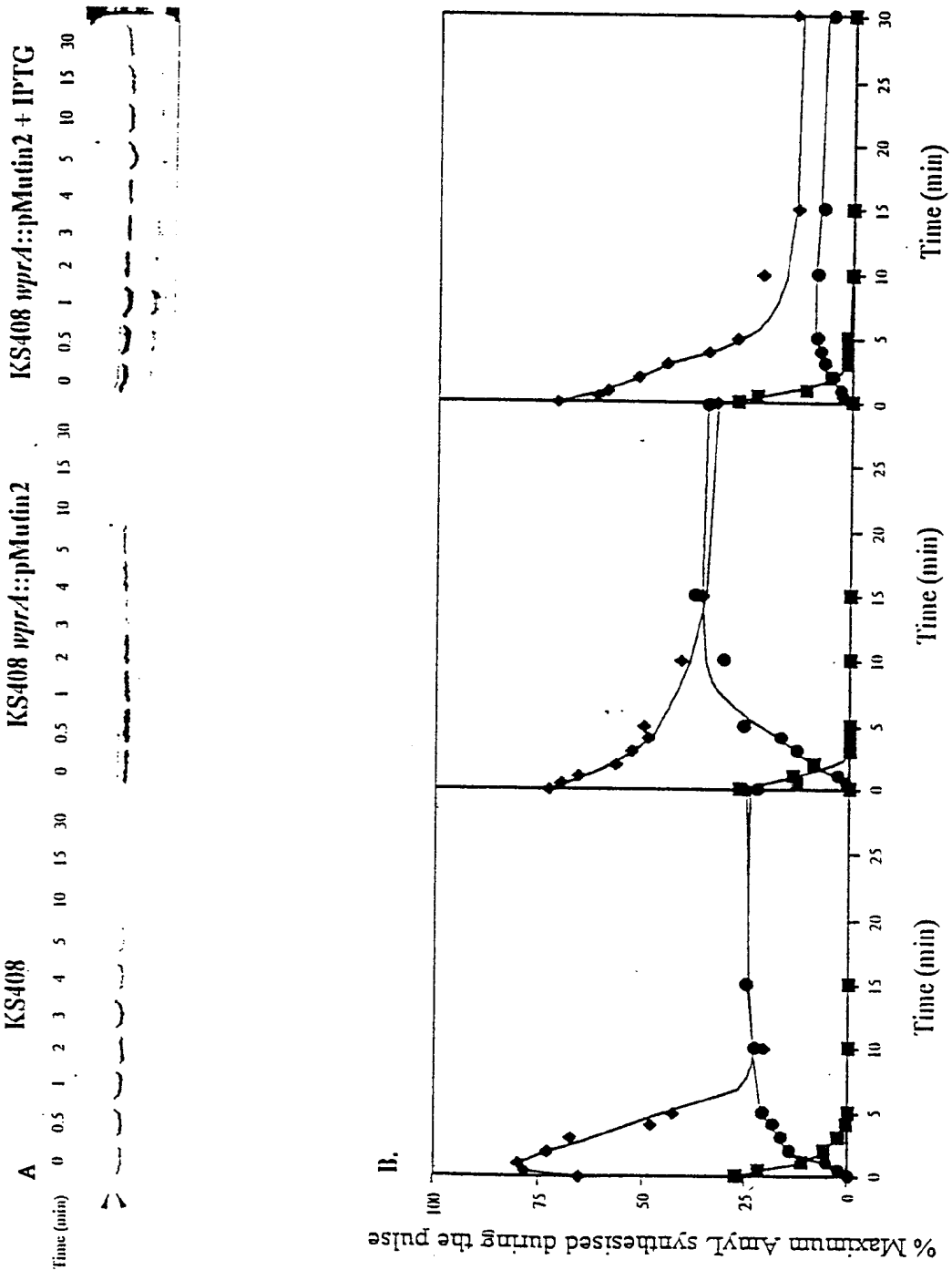

A
KS408
KS408 wprA::pMutin2
KS408 wprA::pMutin2 + IPTG
Time (min)
0 0.5 1 2 3 4 5 10 15 30
B.
% Maximum AmyL synthesised during the pulse
100
75
50
25
0
Time (min)
0 5 10 15 20 25 30

Figure 5

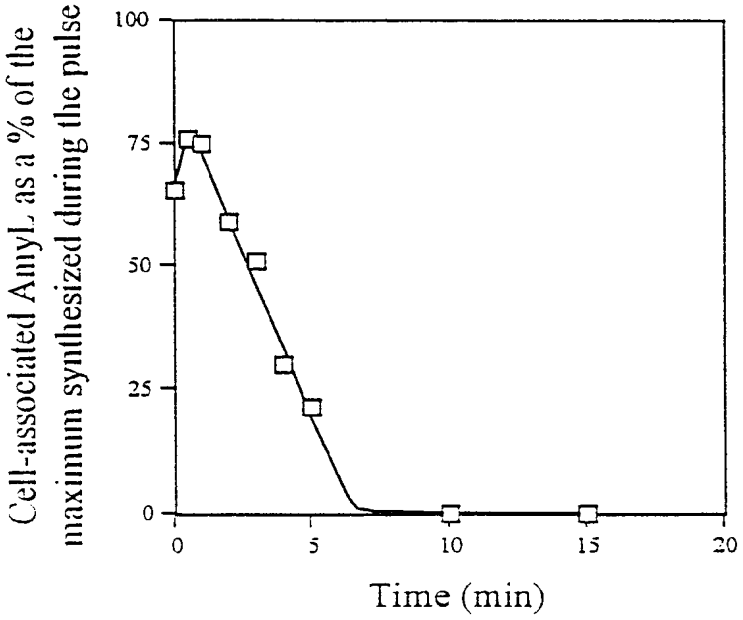
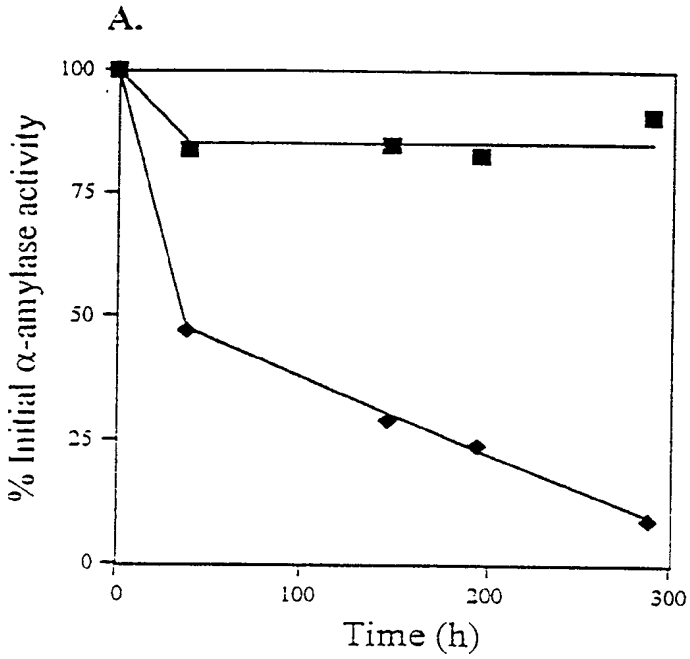
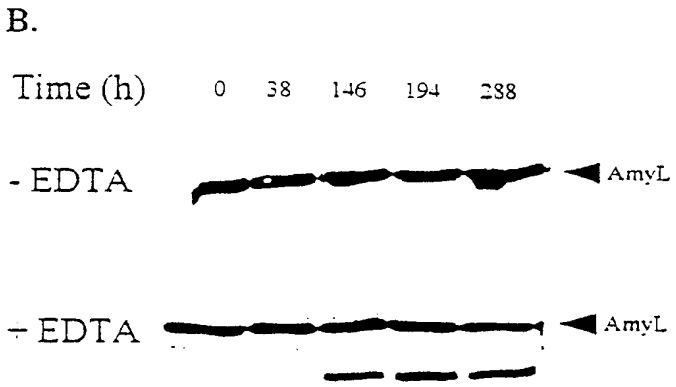

Cell-associated AmyL as a % of the maximum synthesized during the pulse
100
75
50
25
0
0
5
10
15
20
Time (min)

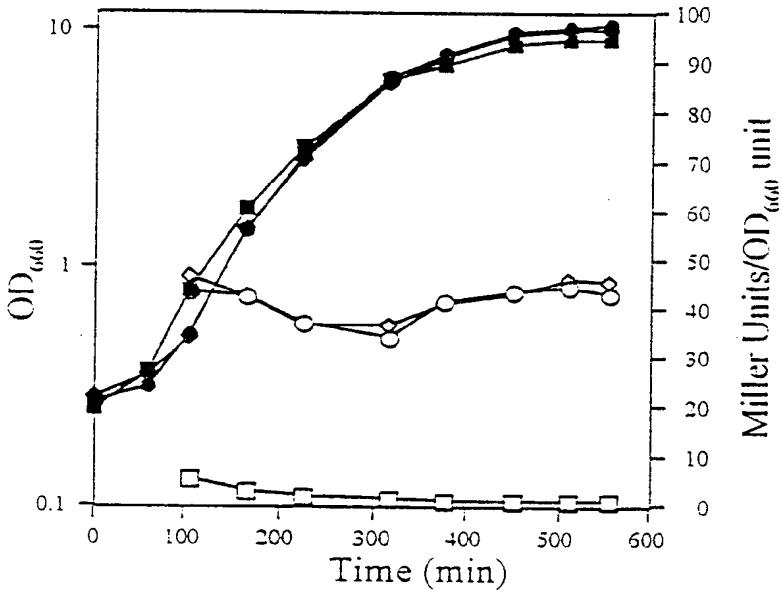
Figure 6


A.
100
75
50
25
0
% Initial α-amylase activity
0
100
200
300
Time (h)


B.
Time (h)
0
38
146
194
288
- EDTA
AmyL
+ EDTA
AmyL

12/12

Figure 7



INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/01051

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/75 C12N9/28 C12N1/21 C12R1/125 //C12N9/56

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MARGOT P ET AL: "The wprA gene of Bacillus subtilis 168, expressed during exponential growth, encodes a cell-wall-associated protease." MICROBIOLOGY (READING) 142 (12). 1996. 3437-3444. ISSN: 1350-0872, XP002075685 see the whole document ---	1-24
Y	SIMONEN M ET AL: "PROTEIN SECRETION IN BACILLUS SPECIES" MICROBIOLOGICAL REVIEWS, vol. 57, no. 1, 1 March 1993, pages 109-137, XP000562630 see page 121, right-hand column - page 122, left-hand column --- -/--	1-24

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 August 1998	09/09/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Van der Schaal, C

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/01051

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 369 817 A (BIOTEKNIKA INTERNATIONAL) 23 May 1990 see the whole document	1-24
Y	WO 92 16642 A (OMNIGENE INC) 1 October 1992 see the whole document	1-24

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INTERNATIONAL SEARCH REPORT

Information on patent family members

National Application No
PCT/GB 98/01051

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0369817 A	23-05-1990	AT 137265 T	15-05-1996
		AU 4476689 A	24-05-1990
		CA 2003078 A	18-05-1990
		DE 68926333 D	30-05-1996
		DE 68926333 T	28-11-1996
		DK 577989 A	19-05-1990
		JP 3067582 A	22-03-1991
		US 5589383 A	31-12-1996
		US 5620880 A	15-04-1997
WO 9216642 A	01-10-1992	US 5294542 A	15-03-1994
		EP 0576606 A	05-01-1994